

Original Research Article

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In vitro Studies on Potentiation of Enrofloxacin by Phytochemicals

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Enrofloxacin, an antimicrobial fluoroquinolone is most commonly used against majority of gram negative bacterial and mycoplasma infections in majority of livestock. Indiscriminate usage of enrofloxacin in clinical practice leads to resistance development to this quinolone drug. Among the various pathways of resistance, efflux pump mediated drug resistance is one of the important pathways identified in the recent past. Phytochemicals namely, theobromine, glycyrrhetic acid and glycyrrhizic acid and capsaicin were identified as efflux pump inhibitors. Phytochemicals which possess efflux pump inhibitory activity if combined with classical antimicrobial agents reduces the development of resistance and also improves their therapeutic efficacy. Interaction between enrofloxacin, and capsaicin, theobromine, glycyrrhetic acid and glycyrrhizic acid were studied by determining MIC and MBC against *E.coli*, *S.aureus*, *K.pneumoniae* and *P.aeruginosa* following CLSI guidelines. There is a significant decrease in MIC and MBC values of enrofloxacin in the presence of phytochemicals. In conclusion, Synergistic interaction of efflux protein inhibitory phytochemicals capsaicin, theobromine, glycyrrhetic acid and glycyrrhizic acid with enrofloxacin was noticed in minimum inhibitory concentration (MIC) and minimum bactericidal concentration.

Introduction

Bacterial infections are one of the major clinical implications observed in routine veterinary practice. From the dawn of civilisation so many drugs, antibiotics came into role to treat bacterial infections. Even after the discovery of many antibiotics starting from penicillins to quinolones, treating the bacterial infections has became a challenging issue till to date due to the emergence of resistance by the bacteria to

antibacterial agents. Antibiotics and other are used as growth promoters, coccidiostats and various purposes for the production of animal meat for human consumption.

Due to over the counter usage, indiscriminate application, failure of following the dosage regimen will sensitize the bacteria to develop resistance. Among several means of resistance development by the bacteria, efflux mediated resistance is of prime importance.

Efflux proteins are present on bacterial cell membranes whose role is extrusion of the antibacterial agents inside the bacterial cell to outside, thereby enhancing their survivability (Borges Walmsley *et al.*, 2003).

Antibiotic resistance is a one health challenge globally. de Kraker *et al.*, (2016), reported that 10 million people will die due to AMR by 2050, if the challenge was not encountered.

Enrofloxacin, a fluoroquinolone antimicrobial is effective against a broad spectrum of gram-positive and gram-negative bacteria. Resistance has developed to this agent because of indiscriminate application (Adamson *et al.*, 2015; Gouvea *et al.*, 2015). It has also been reported that combination of putative efflux pump inhibitors trimethoprim and sertraline with levofloxacin resulted in enhanced therapeutic efficacy of levofloxacin a quinolone antimicrobial drug against *P.aeruginosa* that over expresses *Nex-AB-Oprm*, *MexCD-OprJ* and *MexEF OprM* efflux pumps (Adamson *et al.*, 2015).

Perusal of available literature indicated that there are no reports of usage of phytochemicals that possess efflux inhibitory activity in combination with enrofloxacin to reduce its resistance to microbes are available.

Keeping the background in view, the present study was designed to determine the antibacterial action of enrofloxacin alone and in combination with phytochemicals capsaicin, theobromine, glycyrrhetic acid and glycyrrhizic acid against *S. aureus* ATCC 25923, *E.coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853.

Materials and Methods

Chemicals

Enrofloxacin, Capsaicin, glycyrrhizic acid, glycyrrhetic acid and theobromine were

procured from Sigma-Aldrich, St. Louis, MO, USA. Mueller-Hinton Broth was obtained from M/s Hi Media Laboratories Pvt. Ltd. Mumbai, India. Magnesium chloride was obtained from M/S Fisher Scientific, Mumbai, India and calcium chloride was from M/s SD Fine - Chem Ltd, Mumbai, India. *p*-Iodonitrotetrazolium (INT) was procured from M/s SRL, Mumbai, India. *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *K. pneumoniae* ATCC700603 and *P. aeruginosa* ATCC27853 cultures were procured from Principal investigator, RKVY project, Dept of Veterinary pharmacology and toxicology, NTR CVSc, Gannavaram, SVVU.

Determination of Minimum Inhibitory Concentration (MIC)

Preparation of 0.5 McFarland turbidity standards

Stock solutions of 0.18 M (0.36 N) H_2SO_4 (1% v/v) and 0.048 M BaCl_2 (1.175% w/v $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) were prepared. With a constant stirring to maintain a suspension, 0.5 mL of the BaCl_2 solution was added to 99.5 mL of the H_2SO_4 stock solution. The correct density of the turbidity standard was verified by measuring absorbance using a spectrophotometer with a 1 cm light path and matched cuvettes. The absorbance at 625 nm was 0.08 to 0.13 for the 0.5 McFarland standard. 5 mL aliquots of BaSO_4 were transferred into screw cap tubes of the same size as those used for standardizing the bacterial inoculum (CLSI, 2012).

Preparation of supplements and media cation stock solutions

Stock solution of 10 mg of Mg^{++}/ml was prepared by dissolving 8.36 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 100 ml of deionized distilled water and stock solution of 10 mg of Ca^{++}/ml was prepared by dissolving 3.68 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

in 100 ml of deionized distilled water. They were sterilized by membrane filtration and stored at 2 to 8°C (CLSI, 2012).

Preparation of Cation-Adjusted Muller-Hilton Broth (CAMHB)

Two hundred ml of Muller-Hilton Broth was prepared according to manufacturer's recommendations, autoclaved and chilled overnight at 2 to 8°C. To this chilled broth, 0.2 ml of MgCl₂ stock solution was added with constant stirring followed by addition of 0.4 ml of CaCl₂ stock solution so that the final concentration of Mg and Ca ions in the broth was 10 and 20 mg/l, respectively. The pH of the broth after addition of cations was 7.2 to 7.4.

MIC by broth microdilution method

The broth microdilution method was used to determine the MIC of enrofloxacin against *S.aureus* ATCC 25923, *E. coli* ATCC25922, *K. pneumoniae* ATCC700603 and *P. aeruginosa* ATCC27853. Working standard of 1 µg/ml enrofloxacin was prepared by diluting the stock solution with normal saline. Two-fold serial dilution of enrofloxacin in CAMHB was prepared in 96 well microtiter plate, so that final volume in each well was 100 µl. The bacterial culture incubated in CAMHB at 37±1°C for 6 to 8 h was taken and its turbidity was adjusted to 0.5 McFarland turbidity standard (1 X 10⁸ CFU/ml) which was then diluted 1:20 in CAMHB. When 0.01 ml of this suspension was inoculated into the broth, the final concentration of bacteria was approximately 5 X 10⁵ CFU/ml (range 2 - 8X 10⁵ CFU/ml or 5 X 10⁴ CFU/well). Each plate was sealed properly to prevent drying during incubation. Inoculated microdilution trays were then incubated at 35±2°C for 16 to 20 h in an ambient air incubator.

MIC End Point

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the microdilution wells as detected by the unaided eye or microplate reader (Multiskan™ GO, ThermoFisherScientific™) to discern growth in the wells.

The amount of growth in the wells containing antimicrobial agent was compared with that of growth-control wells (no antimicrobial agent) used in each set of tests. Alternatively, bacterial growth and inhibition was detected by adding 25 µl of INT to each well and incubation for 30 min at 35±2°C. INT is reduced to a red formazan compound by biologically active organisms. Bacterial growth was considered to be inhibited when the solution in the well remained clear. Solvent controls and growth controls were included in each experiment (CLSI, 2012).

Determination of Minimum Bactericidal Concentration (MBC)

The minimum concentration of the drug required to kill >99.9% viable organism after incubation for a fixed length of time (24 hr) under a given set of conditions is known as minimum bactericidal concentration (MBC). The wells that didn't show any visible growth of the organism on MIC microtiter plate were transferred to Mueller Hilton Agar. Then, the plate was incubated for 24 hours at 37°C (CLSI, 2012). MBC values were taken at the lowest concentration that does not show any growth in subculture agar.

Results and Discussion

The mean MIC, MBC values of enrofloxacin alone and in the presence of phytochemicals were depicted in table 1 and 2.

Table.1 Minimum Inhibitory Concentration (MIC, $\mu\text{g}/\text{ml}$) of enrofloxacin alone and in combination with efflux protein inhibitors against the selected bacteria

Name of the test compound	<i>S. aureus</i> ATCC25923	<i>E. coli</i> ATCC25922	<i>K. pneumoniae</i> ATCC700603	<i>P. aeruginosa</i> ATCC27853
Enrofloxacin	0.202	0.020	1.650	2.433
Capsaicin + Enrofloxacin	0.090	0.012	0.266	0.404
Theobromine+ Enrofloxacin	0.110	0.012	0.258	0.450
Glycyrrhetic acid + Enrofloxacin	0.041	0.012	0.404	0.450
Glycyrrhizic acid+ Enrofloxacin	0.11	0.012	0.404	0.450

The values are expressed as mean of six replications

Table.2 Minimum Bactericidal Concentrations (MBC, $\mu\text{g}.\text{ml}^{-1}$) of Enrofloxacin alone and in combination with Efflux protein inhibitors against the selected bacteria

Name of the test compound	<i>S. aureus</i> ATCC25923	<i>E. coli</i> ATCC25922	<i>K. pneumoniae</i> ATCC700603	<i>P. aeruginosa</i> ATCC27853
Enrofloxacin	0.403	0.040	3.300	4.867
Capsaicin+ Enrofloxacin	0.181	0.024	0.532	0.808
Theobromine+ Enrofloxacin	0.220	0.024	0.517	0.900
Glycyrrhetic acid+Enrofloxacin	0.081	0.024	0.808	0.900
Glycyrrhizic acid+Enrofloxacin	0.220	0.024	0.808	0.900

The values are expressed as mean of six replications

The interaction of enrofloxacin and enrofloxacin with efflux protein inhibitors *viz.* capsaicin, glycyrrhizic acid, glycyrrhetic acid and theobromine with regards to MIC and MBC was explored against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 strains and were tabulated in table 1 and 2. The MIC value of enrofloxacin was $0.02 \mu\text{g}.\text{ml}^{-1}$ against *E. coli* ATCC 25922 that was lowered ($0.012 \mu\text{g}.\text{ml}^{-1}$) in presence of capsaicin, glycyrrhizic acid, glycyrrhetic acid and theobromine resulting in 40% improvement in MIC against

E. coli. The MIC value of $0.2 \mu\text{g}.\text{ml}^{-1}$ for enrofloxacin alone was observed against *S. aureus* ATCC 25923 which was lowered by 55% in the presence of capsaicin and 45% in presence of glycyrrhizic acid, glycyrrhetic acid and theobromine. Similarly, it was shown that capsaicin potentiates enrofloxacin against *S. aureus* due to the inhibitory effect on NorA efflux pump of *S. aureus* (Kalia *et al.*, 2012). The MIC value of enrofloxacin against *K. pneumoniae* ATCC 700603 was $1.650 \mu\text{g}.\text{ml}^{-1}$ which was reduced by 83.8% in the presence of capsaicin and was reduced to 75% in the presence of glycyrrhizic acid,

glycyrrhetic acid and theobromine. The MIC value of enrofloxacin against *P. aeruginosa* ATCC 27853 was $2.433 \mu\text{g.ml}^{-1}$ which was reduced to 83% in the presence of capsaicin, glycyrrhizic acid, glycyrrhetic acid and theobromine. It was also reported that phytochemicals like plumbagin, NDGA, and shikonin were able to increase susceptibility of bacterial organisms to antibiotics and toxic compounds and were also the most efficient in inhibiting AcrB-mediated substrate efflux in bacteria (Ohene-Agyei *et al.*, 2014).

The MBC value of enrofloxacin against *E.coli* ATCC 25922 was $0.041 \mu\text{g.ml}^{-1}$. The MBC values of enrofloxacin alone and in the presence of efflux protein inhibitors was presented in table 2. The MBC of enrofloxacin against *E.coli* was reduced to $0.024 \mu\text{g.ml}^{-1}$ in the presence of capsaicin, glycyrrhizic acid, glycyrrhetic acid and theobromine. The MBC value of enrofloxacin against *S.aureus* ATCC 25923 was $0.403 \mu\text{g.ml}^{-1}$, which was reduced to $0.181 \mu\text{g.ml}^{-1}$ in the presence of capsaicin and to $0.220 \mu\text{g.ml}^{-1}$ in the presence of glycyrrhizic acid and theobromine. The MBC value of enrofloxacin against *K.pneumoniae* ATCC 700603 was $3.300 \mu\text{g.ml}^{-1}$ which was reduced to $0.532 \mu\text{g.ml}^{-1}$ in the presence of capsaicin and was reduced to $0.517 \mu\text{g.ml}^{-1}$ in the presence of theobromine. The MBC value of enrofloxacin was reduced to $0.808 \mu\text{g.ml}^{-1}$ in the presence of glycyrrhizic acid and glycyrrhetic acid. The MBC value of enrofloxacin against *P.aeruginosa* ATCC 27853 was $4.867 \mu\text{g.ml}^{-1}$ which was reduced to $0.808 \mu\text{g.ml}^{-1}$ in the presence of capsaicin and to $0.900 \mu\text{g.ml}^{-1}$ in the presence of glycyrrhizic acid, glycyrrhetic acid and theobromine. The results together suggest that the presence of efflux protein inhibitors enhances the bactericidal effect, which may be due to their efflux protein inhibitory potential; as a result they indirectly increase the intracellular concentration of the antimicrobial agent.

Based on the above results it can be concluded that phytochemicals as such may not show antibacterial action comparable to antibiotic enrofloxacin, but in combination they enhanced the antibacterial activity which can be utilised to reduce the amount of antibiotic required to produce the therapeutic effect. It can reduce the resistance development as well as cost of therapy.

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